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POST-MORTEM PHARMACOGENETICS

CITALOPRAM-POSITIVE SUICIDES AS A MODEL POPULATION

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*Coming together is the beginning.
Keeping together is progress.
Working together is success.*

- Henry Ford

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Rahikainen AL, Palo JU, de Leeuw W, Budowle B, Sajantila A. DNA quality and quantity from up to 16 years old post-mortem blood stored on FTA cards. *Forensic Sci Int.* 2016 Apr; 261:148-53.
- II Rahikainen AL, Majaharju S, Haukka J, Palo JU, Sajantila A. Serotonergic *5HTTLPR*/rs25531 s-allele homozygosity associates with violent suicides in male citalopram users. *Am J Med Genet B Neuropsychiatr. Genet.* 2017 Oct; 174(7): 691-700.
- III Rahikainen AL, Palo JU, Haukka J, Sajantila A. Post-mortem analysis of suicide victims reveals *ABCB1* haplotype 1236T-2677T-3435T as a candidate predisposing factor behind adverse drug reactions in females. *Pharmacogenet Genomics.* 2018 Apr; 28(4):99-106.
- IV Rahikainen AL, Vauhkonen P, Pett H, Palo JU, Haukka J, Ojanperä I, Niemi M, Sajantila A. Completed suicides of citalopram users - the role of *CYP* genotype and adverse drug interactions. *Int J Legal Med.* 2018; Published online September 1, DOI: 10.1007/s00414-018-1927-0.

Electronic supplementary material is included in this thesis.

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

ADME	Absorption, distribution, metabolism, and excretion
ADR	Adverse drug reaction
BAC	Blood alcohol concentration
BBB	Blood-brain barrier
Bp	Base pair
CE	Capillary electrophoresis
CI	Confidence interval
CNV	Copy number variation
CoD	Cause of death
DNA	Deoxyribonucleic acid
CYP2C19	Cytochrome P450, family 2, subfamily C, polypeptide 19
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6
E.g.	Exempli gratia (for example)
EM	Extensive metabolizer
gMP	Genetically predicted metabolizer phenotype
5-HT	5-hydroxytryptamine (serotonin)
HTR2B	5-hydroxytryptamine receptor 2B
5HTTLPR	Serotonin transporter gene-linked promotor region
ICD-10	International classification of disease, 10 th revision
I.e.	Id est (that is)
IM	Intermediate metabolizer
MAOA	Monoamine oxidase A
MoD	Manner of death
OR	Odds ratio
PCR	Polymerase chain reaction
P-gp	Permeability glycoprotein
PM	Poor metabolizer
RT-PCR	Real-time polymerase chain reaction
SERT	Serotonin transporter
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
STR	Short tandem repeat
UM	Ultrarapid metabolizer
uVNTR	Upstream variable number of tandem repeats
Vs.	Versus

1 ABSTRACT

The functionality of a drug depends on its dose. “The right dose, for the right patient, at the right time” has been a long-standing principle in medicine. With too low of a dose, the desired response is not attained: with too high of a dose unwanted side effects, and even death, may occur.

In most drugs, the dose is not individually tailored. A standard dose is effective for most users but may result in an insufficient response or severe side effects for a certain population of users. Only recently has personalized drug therapy become more feasible due to an understanding of the genetic factors behind the fate of a drug in human body. Most of this progress is due to the emerging field of pharmacogenetics and pharmacogenomics.

Genetic variation may shape drug response in multiple ways and pharmacogenetic analysis prior to drug initiation can help prevent unwanted outcomes. Depression is a known risk factor for suicide. Antidepressants, used to treat depression, are a good example of drugs in which the effective dose and correct plasma level should be attained shortly after initiation of the drug.

The most widely used antidepressants worldwide are currently selective serotonin reuptake inhibitors (SSRIs); citalopram and escitalopram are the most-sold SSRIs in Finland. Although they are acutely rather non-toxic, serious adverse effects may occur in a dose-dependent manner in case of poor metabolism capacity or due to drug-drug interactions. A noteworthy problem of SSRIs is insufficient treatment response. It has been suggested that ultrarapid metabolism is one factor behind this unfavorable phenomenon.

In this study, citalopram-positive completed suicide cases were investigated. It was hypothesized that among these cases, considered an extreme population of antidepressant users, pharmacogenetic factors affecting the treatment success of SSRIs would be enriched.

Post-mortem material may pose a challenge for genetic analysis. For this reason, the material used for the study (blood stored on FTA cards) was characterized for concentration and degradation status. After this baseline work, FTA samples from suicide cases were genotyped for pharmacogenetically relevant gene polymorphisms. In particular, variants that affect citalopram pharmacodynamics and pharmacokinetics and variants that affect serotonin metabolism were concentrated. In the genotyping analyses, fragment length analysis, Sanger sequencing, and TaqMan chemistry-based methods were used. The role of drug interactions and adverse drug effects in suicide deaths was also evaluated. Serotonergic and arrhythmic adverse effects received special attention, as citalopram itself holds potential for these types of adversities.

It was found that DNA is rather well preserved over time in a FTA card matrix. Thus, in addition to highly sensitive forensic analysis, pharmacogenetic analysis was also possible using this material. However,

time-dependent DNA degradation does occur and has an effect when larger DNA fragments are amplified.

As a main result, pharmacogenetic analysis revealed enrichment of a combined group of genetically predicted extreme *CYP2C19* phenotypes (ultrarapid and poor metabolizers) among citalopram-positive suicide cases compared to Finnish population controls. This was an interesting finding, as it may indicate a higher incidence of poor treatment efficacy or elevated toxicity of the antidepressant among suicide victims. Also, a trend between increased post-mortem citalopram concentration and genetically predicted *CYP2C19* phenotype was observed. This trend needs to be investigated further in larger sample set. In the interaction analysis, several drug interactions and adverse effects were observed. In approximately 8% of drug poisoning cases, the detected adversity was evaluated to have a significant contribution to the fatal outcome.

Based on the obtained results, pharmacogenetic testing prior to initiation of SSRIs could be beneficial. In a medico-legal context, pharmacogenetic analysis could elucidate the manner of death (suicide vs. accidental death) in drug poisoning cases, especially if the background information is obscure. It also appears that the role of drug interactions is currently underestimated. These findings suggest that the evaluation of post-mortem toxicological results and determination of manner of death could be improved by developing tools that detect interacting drug pairs and call attention to drug metabolism affected by pharmacogenetic variation.

2 INTRODUCTION

Medico-legal investigations provide a unique source of data to study unexpected natural and unnatural deaths (e.g., suicides). The information obtained from post-mortem investigations can reveal patterns and trends, which can ultimately lead to preventive acts. The National Suicide Prevention Project in Finland, carried out from 1986 to 1996, is an example of a multidisciplinary collaboration combining medical, social, and public health expertise (Lönnqvist et al. 1993; Lönnqvist et al. 1995). After this project, suicide deaths showed clear decreasing trend; in 1990 the suicide rate was 30.2 per 100 000 individuals (49.2 for males and 12.4 for females), whereas in 2016 the rate was 14.3 (22.7 for males and 6.2 for females). The decrease is especially notable in males.

Mental disorders are a background factor in the majority of suicide deaths (Henriksson et al. 1993; Lönnqvist et al. 1995). Female victims suffer more commonly from depressive disorders and males from substance-use related problems and personality disorders (Arsenault-Lapierre et al. 2004). Indeed, the cause of death (CoD) register data (Official Statistics of Finland 2010) contrasted to the antidepressant consumption data (Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2016) suggests a correlation between increased antidepressant usage and decrease in suicide rates.

It is known that not all users benefit from antidepressive medication (Barak et al. 2011; Finnish Medicines Agency (Fimea) 2013; Jukic et al. 2018). The reasons behind this phenomenon are unknown. As antidepressant usage as such seems to have a beneficial effect on the number of suicides (Haukka et al. 2009), it is essential to understand the factors behind treatment failures. By identifying these factors, which may be partly genetic, it may be possible to further decrease the number of suicides.

Previous studies have shown that inter-individual variation in drug response can result from pharmacogenetic factors (i.e., genetic variation in genes encoding drug-metabolizing enzymes, transporters, and pharmacodynamic targets of drugs) (Ingelman-Sundberg et al. 2007; Peters et al. 2004). If pharmacogenetic factors are involved in antidepressant treatment failures and thus contribute to suicide deaths, these factors should be revealed by investigating medico-legal autopsy material, such as citalopram-positive suicide deaths. In addition to the preventive aspect, pharmacogenetic analysis can produce useful information for the medico-legal field and possibly facilitate the interpretation of post-mortem toxicological results. With this information one may be able to answer questions such as “How significant of a role can poor metabolism capacity have in elevated post-mortem drug

concentrations?” or “Could slightly elevated drug concentrations result from genetically compromised drug metabolism rather than intentional overdose?”

3 REVIEW OF THE LITERATURE

3.1 PHARMACOGENETICS

Pharmacogenetics can be defined as the study of the influence of inherited factors on drug response (Vogel F 1959). Pharmacogenetic studies aim to understand how genetic differences between individuals modify the obtained drug response and the drug dose required. By using this knowledge, personalized treatments with proper dose, minimal side effects, and optimal response can be attained.

The roots of pharmacogenetics are in the 1950s. In 1952, primaquine, a drug used to treat malaria, was observed to induce hemolytic anemia in some patients (Hockwald et al. 1952). The patients were found to suffer from glucose-6-phosphate dehydrogenase deficiency, leading to primaquine sensitivity and the observed adverse reaction (Carson et al. 1956). Almost simultaneously, inter-individual variation was also observed among other drugs, such as isoniazid (Evans et al. 1960) and succinylcholine (Goedde et al. 1968; Kalow and Staron 1957). However, the molecular-level processes behind adverse drug reactions (ADRs) were not revealed until the 1980s when a genetic defect for a cytochrome P450 (CYP) 2D6 enzyme, affecting debrisoquine metabolism, was characterized (Gonzalez et al. 1988).

Genetic variation may affect drug response in multiple ways. While defects in drug pharmacokinetics are the most investigated aspects, pharmacodynamic factors are also relevant (Ingelman-Sundberg et al. 2007).

Pharmacokinetics refers to individual's ability to absorb, distribute, metabolize, and excrete foreign compounds. These processes can be abbreviated as ADME processes (Absorption, Distribution, Metabolism, and Excretion). The purpose is to protect the body against harmful, exogenous compounds. Thus, when a xenobiotic (e.g., a drug) is absorbed into the body it is rapidly modified by various enzymes with phase I (phase I metabolism) and phase II reactions (phase II metabolism). The aim of these reactions is to modify the lipophilic compound to be more hydrophilic, and thus facilitate its excretion. In phase I reactions, the compound is modified to be more reactive by exposing or introducing polar groups. In phase II, highly polar groups (e.g., glucuronic acid) are introduced into the original compound or to the phase I metabolite.

The processes mentioned above define the outcome of drug treatment as well. Depending on the nature of the compound (active drug vs. pro-drug), individual differences in metabolic capacity can lead to different outcomes such as inadequate treatment response or ADRs. Inadequate treatment response may result from ultrarapid metabolism (active drug) (Bertilsson et al. 1985; Bertilsson et al. 1993) or from poor metabolism (pro-drug) (Sindrup et al. 1990). ADRs may be either caused by poor metabolism capacity

(accumulation of a active drug) (Hamelin et al. 1996) or ultrarapid metabolism capacity (increased activation of pro-drug) (Kirchheiner et al. 2007). In addition to metabolic processes, inter-individual variation in drug transporter genes can affect drug response, as multiple transporters participate in absorption and excretion processes (Shitara et al. 2006).

Pharmacodynamics refers to the actual effect of a drug, namely its interaction with the target molecule. A good example of a drug therapy where both pharmacokinetic and pharmacodynamic factors are tested is anticoagulant warfarin therapy. The treatment outcome of warfarin is a result of its metabolism (catalyzed by CYP2C9 enzyme) and the expression level of its pharmacodynamic target protein (vitamin K epoxide reductase (VKOR)). Individuals with certain genetic variants of *CYP2C9* and *VKOR* are considered “warfarin sensitive” as they are in danger of over-anticoagulation, an adverse effect of warfarin. Among warfarin-sensitive individuals, hemorrhagic complications are possible with the standard dose used. Despite availability, pharmacogenetic testing prior to initiation of warfarin medication is not currently in routine use (Johnson et al. 2017).

3.1.1 PHARMACOGENETICS IN A MEDICO-LEGAL CONTEXT

Compared to clinical medicine, pharmacogenetics was introduced into a post-mortem context far later (Druid et al. 1999; Levo et al. 2003; Pajarinen et al. 1996; Saltee et al. 2000; Savolainen et al. 1997). As genetic variation in genes encoding drug-metabolizing enzymes can lead to severe ADRs and can even cause fatal intoxications (Kupiec et al. 2006; Launiainen et al. 2010; Lazarou et al. 1998; Madadi et al. 2010; Musshoff et al. 2010; Neukamm et al. 2013; Sajantila et al. 2010), post-mortem pharmacogenetics can offer a useful tool for the medico-legal field.

Compared to clinical pharmacogenetics, post-mortem aims are different. First, as post-mortem research is accomplished in a selected population (i.e., deaths investigated by medico-legal autopsy), patterns and trends that ultimately can lead to preventive acts can be revealed. Second, post-mortem pharmacogenetics can provide defined information for interpretation of the forensic toxicological results (Druid et al. 1999; Fonseca et al. 2016; Koski et al. 2007; Koski et al. 2006; Levo et al. 2003). A more detailed interpretation may further direct the manner of death (MoD) determination (Koren et al. 2006; Koski et al. 2007). Without pharmacogenetic knowledge it can sometimes be difficult to differentiate between accidental or intentional poisoning deaths, especially when background information is scarce. Although post-mortem pharmacogenetics could serve as a useful tool for post-mortem toxicology and direct the MoD decision in certain deaths, it is not currently part of routine investigations.

3.2 MEDICO-LEGAL INVESTIGATIONS IN FINLAND

According to the Finnish law (1973/459, 7§), a medico-legal cause of death investigation (CoD) is initiated by the police if 1) death is not known to be related to a disease or the deceased has not received medical treatment during the last illness; 2) death is presumed to be related to an accident, homicide, suicide, occupational disease, medical treatment, or war; or 3) it was otherwise unexpected. At the end of the investigation, the cause and manner of death is determined by a forensic pathologist. In 2016, approximately 16% of all deaths in Finland underwent a medico-legal autopsy (Official Statistics of Finland 2016a); the rate was even higher in the past (24% in 2009).

Based on the facts mentioned above, a suspected suicide always leads to a medico-legal investigation. Statistics relating to suicide deaths can thus be considered exhaustive and reliable (Ylijoki-Sorensen et al. 2014). One of the routine procedures in a medico-legal autopsy is to collect samples for forensic toxicological analysis. During the years 2014 to 2016 forensic toxicological analysis was performed in over 70% of medico-legal autopsies (personal communication, Dr. Pirkko Kriikku).

Post-mortem toxicology is an invaluable tool when a possibility of fatal drug poisoning is evaluated. The interpretation of the results, however, demands special expertise as post-mortem drug concentrations are site- (cardiac vs. femoral blood) and time-dependent (Pounder and Jones 1990; Prouty and Anderson 1990). By collecting different samples (e.g., blood, urine, vitreous humor, and hair), the time of drug consumption, acute ingestion vs. continuous use, can be estimated. Parent drug-metabolite ratios can also reveal if the drug consumption was acute or chronic (Druid and Holmgren 1997; Koski et al. 2007).

In suicides, the MoD is determined based on the underlying CoD and all further information available (patient records, post-mortem toxicology report, records of death circumstances, and evidence of intent e.g., farewell letter). If the additional information is scarce it can be difficult to determine if the death was intentional or unintentional (i.e., an accident). According to the World Health Organization (WHO), a suicidal act should be defined as “self-infliction of injury with varying degrees of lethal intent and awareness of motive” and suicide as “suicidal act with fatal outcome” (World Health Organization 1968). Thus, cases with uncertainty of intention should be classified as “undetermined” deaths (Lunetta et al. 2002). The majority of these undetermined deaths result from fatal poisonings (Öhberg and Lönnqvist 1998).

A good example of a medico-legal case where the MoD was defined to be undetermined (i.e., the possibility of suicide could not be excluded) was reported by Koski et al. (Koski et al. 2007). In that study, post-mortem toxicological findings were elegantly combined to pharmacogenetic data. Koski et al. (2007) studied the tricyclic antidepressant doxepin, and observed that poor metabolism capacity due to a genetic variation in *CYP2D6* had most

probably contributed to death. The MoD was concluded likely to be accident and not suicide. It was observed that due to the genetic defect, the CYP2D6 enzyme-mediated pathway was not in use and thus the CYP2C19 enzyme-mediated pathway (resulting in nordoxepin) showed elevated metabolite concentrations. The increased parent drug-metabolite ratio revealed non-acute use of drug and directed suspicions towards the metabolic defect of the CYP2D6 enzyme. As mentioned previously, the parent drug-metabolite ratio reveals if the drug consumption was acute or chronic but can also be used to estimate the role of a genetic defect in drug metabolism.

3.2.1 TRENDS IN SUICIDE DEATHS IN 1921-2016 AND ANTIDEPRESSANT CONSUMPTION IN 1990-2016

Statistics Finland maintains the Finnish Cause of Death (CoD) register and collects yearly ICD-10 (World Health Organization 2011) coded data from the death certificates. Suicide mortality has been followed for decades and summaries of suicide rates are provided annually (Figure 1).

The suicide rate in Finland has been rather high compared to other Nordic countries (Titelman et al. 2013). The nationwide “National Suicide Prevention Project in Finland” was carried out in Finland between 1986 and 1996 (Hakanen and Upanne 1996; Lönnqvist et al. 1993). The material was collected within one year (1.4.1987-31.3.1988). All completed suicide cases ($n = 1397$, males $n = 1077$ and females $n = 320$) within the time period were included (Lönnqvist et al. 1993). Suicide cases were investigated with a psychological autopsy method which, in addition to medico-legal autopsy, included interviews of relatives and healthcare professionals.

After the highest suicide peak ($n = 1512$) in 1990, the suicide rate (per 100,000 inhabitants) has been decreasing constantly (Figure 1). In 1990 the rate was 30.2 (49.2 for males and 12.4 for females) and in 2016 ($n = 787$) the rate was 14.3 (22.7 for males and 6.2 for females). The decrease is especially prominent in males. In females there was a decrease but it is not as notable as in males. The reason for this sex difference is currently unknown. In general, the male:female ratio in completed suicides is approximately 3:1 in Finland. Among males, the use of violent methods is more common compared with females, who tend to choose non-violent methods (i.e., self-poisoning) (Denning et al. 2000; Pirkola et al. 2003).

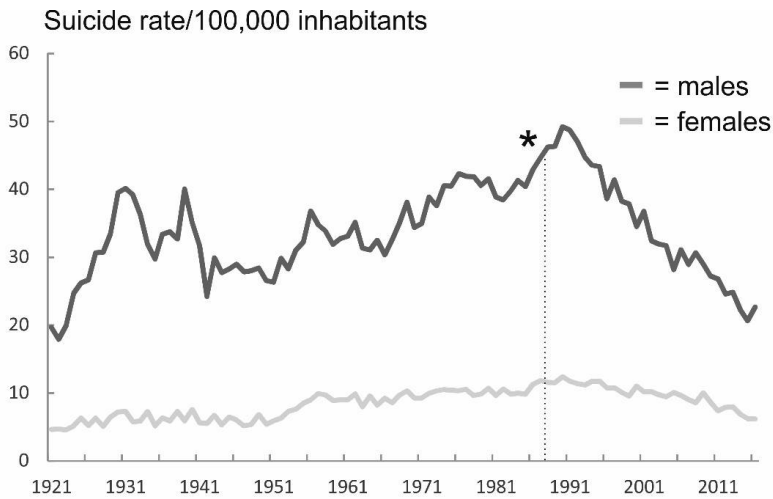


Figure 1 Suicide rates per 100,000 inhabitants in Finland from 1921-2016, stratified by sex (Official Statistics of Finland 2010; Official Statistics of Finland 2011; Official statistics of Finland 2012; Official Statistics of Finland 2013; Official Statistics of Finland 2014; Official Statistics of Finland 2015; Official Statistics of Finland 2016b). The suicide rate was constantly increasing until 1990. Since then the rate has decreased gradually. The National Suicide Prevention Project in Finland took place during 1986-1996. It included three phases: research phase in 1987 (marked with star and dotted line), implementation phase, and evaluation phase (Hakanen and Upanne 1996).

The decreasing number of suicides coincided temporally with the National Suicide Prevention Project in Finland, but the decrease in suicides obviously depends on multiple factors such as the state of the economy, trends in alcohol consumption (Hintikka et al. 1999), and societal changes (Fekete et al. 2001). However, as depressive disorders are known to be a risk factor for suicides (Henriksson et al. 1993) and better recognition of depression was one of the aims of the project, it can be deduced that improved treatment of depression (including increased use of antidepressants) was probably one of the underlying reasons (Figure 2) (Isacsson et al. 1997).

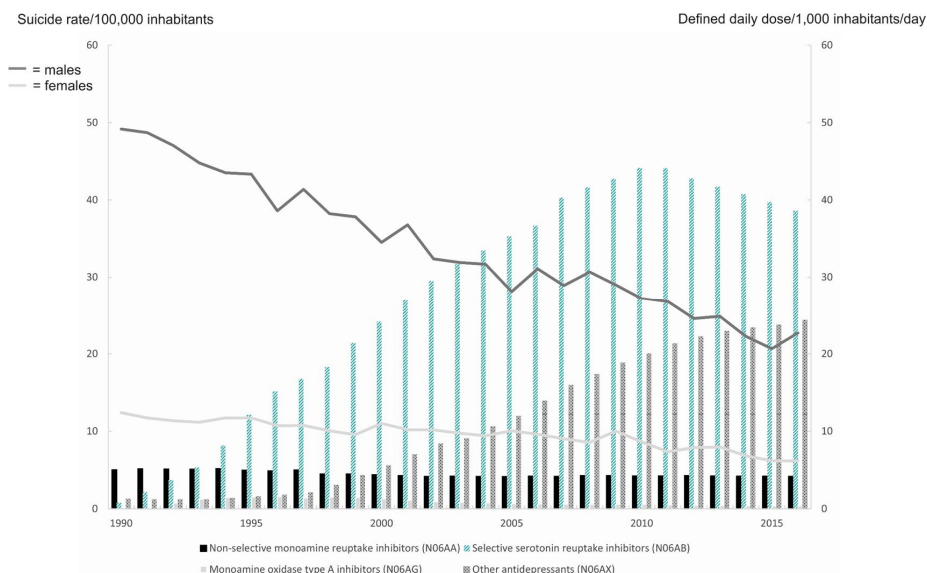


Figure 2 Suicide rates from 1990-2016 (primary axis) (Official Statistics of Finland 2010; Official Statistics of Finland 2011; Official statistics of Finland 2012; Official Statistics of Finland 2013; Official Statistics of Finland 2014; Official Statistics of Finland 2015; Official Statistics of Finland 2016b) vs. antidepressant consumption (secondary axis) (Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2016; Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2017).

3.3 SELECTIVE SEROTONIN REUPTAKE INHIBITORS

Selective serotonin reuptake inhibitors (SSRIs) are currently the most commonly used antidepressants in Finland (Figure 2). According to the Finnish Medicines Agency (Fimea) sales report, the majority of SSRI sales is due to two compounds, citalopram and escitalopram (Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2016; Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2017). The difference between these two drugs is that citalopram is a racemic mixture of pharmacodynamically active and inactive forms of S- and R-enantiomers, respectively. Escitalopram contains only the active S-form (Figure 3).

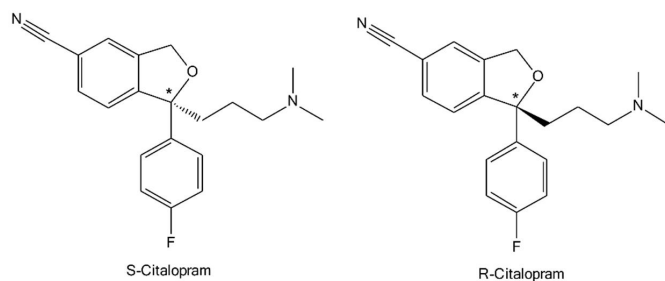


Figure 3 Chemical structure of citalopram enantiomers. The figure was produced with ChemDraw Professional 17.0 (PerkinElmer Informatics).

While the biological background of depression is still unknown, already in the 1950s key observations (Udenfriend et al. 1957) leading to the catecholamine hypothesis and the monoamine theory in the 1960s were reported. These theories suggest that depression results from depletion of serotonin, norepinephrine, and dopamine (Schildkraut 1965). It was proposed that depression could be treated by increasing amount of these key neurotransmitters. However, later studies have shown that this theory alone was far too simplistic, and a more complex chain of events (e.g., neuroplasticity and adult neurogenesis) seem to play a role in treatment response (Castren and Hen 2013; Hyman and Nestler 1996).

SSRIs were invented in the early 1970s (Wong et al. 1974) as the role of low serotonin was highlighted by multiple studies (Ashcroft and Sharman 1960; Shaw et al. 1967; Åsberg et al. 1973). SSRIs increase the amount of serotonin by inhibiting its reuptake from the synaptic cleft to the presynaptic neuron. Prior to SSRIs, tricyclic antidepressants and monoamine oxidase A (MAOA) inhibitors were used to treat depressive symptoms. Due to the less specific mechanism of action (leading to side effects) and narrow therapeutic range (Shaw et al. 1986) these drugs were rapidly replaced by SSRIs. Fluoxetine was the first SSRI on the market (Wong et al. 1974), followed by citalopram a few years later. Escitalopram was introduced most recently (in 2002) and arrived to Finland in 2003 (Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2016). Since then its usage has gradually increased. In 2015 escitalopram usage surpassed that of citalopram. The usage of other SSRIs, namely fluoxetine, sertraline, fluvoxamine, and paroxetine is much lower (Finnish Medicines Agency (Fimea) and Social Insurance Institution of Finland (Kela) 2016).

3.3.1 SSRIS MODIFY THE LEVEL OF KEY NEUROTRANSMITTER SEROTONIN

Serotonin (5-hydroxytryptamine, 5-HT) was first discovered in 1948 from bovine serum and named as serotonin due to its localization and

vasoconstrictor properties (Rapport et al. 1948). A few years later it was also identified from other locations, including the brain (Twarog and Page 1953).

In the central nervous system, serotonin serves as a chemical messenger between nerve cells, or neurons. After neuronal excitation, serotonin is released from the presynaptic neuron to the synaptic cleft. Serotonin diffuses towards the postsynaptic receptors, which in turn deliver the message further. The serotonergic action is terminated by serotonin transporters (SERT or 5-hydroxytryptamine transporter, 5-HTT), the pharmacodynamic targets of SSRIs. By inhibiting this transporter, SSRIs increase the amount of serotonin in the synaptic cleft and thus prolong its effects. This has been associated with alleviation of depressive symptoms.

Although the biochemical change in serotonin levels is rapid after the initiation of SSRIs, the actual treatment response is obtained gradually and takes usually weeks or even months to develop. However, remission and full recovery is not achieved by all users. Some studies suggest that up to 50% of SSRI users feel that they have not benefitted from their initial medication (Barak et al. 2011; Finnish Medicines Agency (Fimea) 2013). The reason for this is unknown.

Among genetic studies evaluating the inter-individual differences in SSRI treatment response, one of the most intensively studied genetic polymorphisms is the serotonin transporter gene-linked promotor region (*5HTTLPR*) polymorphism. This regulatory polymorphism affects the promoter activity of the *5HTT* gene and further the expression level of the transporter protein and ultimately decreases serotonin reuptake. The *5HTTLPR* polymorphism is characterized by two length variants, known as short (s) and long (l). The reduced gene expression is caused by the shorter variant (Heils et al. 1996; Lesch et al. 1996). In addition, the transcriptional efficacy of the *5HTT* gene might be affected by a single nucleotide polymorphism (SNP), rs25531 A>G (Hu et al. 2006; Kraft et al. 2005; Philibert et al. 2008). In previous studies, the ss genotype of *5HTTLPR* has been associated with poor antidepressant treatment response (Serretti et al. 2004; Smeraldi et al. 1998; Yu et al. 2002), development of depression after a stressful life event (Caspi et al. 2003), and suicidality (Bondy et al. 2000; Courtet et al. 2001). However, converse findings have also been published (Kim et al. 2000).

In addition to 5HTT, other potential factors that can affect serotonin levels or otherwise alter the obtained SSRI response are the monoamine oxidase A (MAOA) enzyme and pre- and post-synaptic serotonin receptors (HTRs). The mitochondrial MAOA enzyme is responsible for degradation of serotonin in the presynaptic neuron. Enhanced degradation of serotonin could possibly lower the levels of releasable serotonin and indirectly affect SSRI treatment response. Then again, as serotonin receptors are responsible for both termination of serotonin release and delivering the chemical message further, alterations in these processes could potentially cause inter-individual variation to drug response.

In 2010, a specific *HTR2B* Q20* polymorphism was found (Bevilacqua et al. 2010) with high frequency in the Finnish population but was absent or rare in other populations. This polymorphism causes premature stop-codon formation in the serotonin receptor 2B coding DNA sequence and thus disturbs the correct formation of the *HTR2B* receptor protein. The *HTR2B* Q20* polymorphism has been associated with impulsive violence (Bevilacqua et al. 2010; Bevilacqua and Goldman 2013) and alcohol-related risk behavior (Tikkanen et al. 2015). In *MAOA* there is also a polymorphism, monoamine oxidase A upstream variable number of tandem repeats (*MAOA-uVNTR*) which has been connected to extreme violent behavior (Tiihonen et al. 2015). As impulsive and violent behavior may also precede a suicidal act, it has been speculated that *HTR2B* Q20* and *MAOA-uVNTR* polymorphisms could be involved as background factors and increase the risk for completed suicide, especially in combination with alcohol consumption (Bevilacqua et al. 2010).

3.4 PHARMACOKINETICS OF SSRIS WITH SPECIAL FOCUS ON CITALOPRAM AND ESCITALOPRAM

CYP enzymes

SSRIs are metabolized by cytochrome P450 (CYP) enzymes. In the case of citalopram and escitalopram, the most important metabolic enzyme is CYP2C19. CYP enzymes are responsible for oxidative, phase I metabolism of their substrates. Of the large CYP enzyme family, CYPs from families 1 to 3 are mainly responsible for metabolism of xenobiotics. CYPs from other families metabolize endogenous substances such as steroids and fatty acids. The most important drug metabolism enzymes are CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Tornio and Backman 2018).

CYP enzymes are encoded by *CYP* genes. Human *CYP* genes are named as follows: *CYP* written in italics represents “cytochrome P450” and refers to the gene (standard capital letters referring to protein/enzyme), followed by an Arabic numeral showing a family and after a letter indicating a subfamily. The last Arabic numeral identifies the individual gene within the subfamily (e.g., *CYP2C19*, *CYP2D6*) (Nebert et al. 1987; Nelson et al. 1993; Wain et al. 2002).

CYP genes are highly polymorphic. Due to the large number of variations, a standard star (*)-allele nomenclature (Ingelman-Sundberg et al. 2000) and a frequently updated database has been developed (Gaedigk et al. 2018). After genotyping of the individual variants, the obtained result is translated into a *-allele, which corresponds to haplotype. In this system, the *1-allele is designated as the wild-type allele (defined by the absence of variants among polymorphisms genotyped) and associated with normal enzyme activity. Maternal and paternal *-alleles are then reported as combined diplotype which represents the “*CYP* genotype” (e.g., *CYP2C19* *1/*1).

Based on the defined genotype, the genetically predicted metabolizer phenotype (gMP) can be determined (Gaedigk et al. 2008). In the case of

CYP2C19 and *CYP2D6*, four functional phenotype categories are commonly used; poor metabolizers (PM, no enzyme activity), intermediate metabolizers (IM, decreased enzyme activity), extensive or normal metabolizers (EM, normal enzyme activity), and ultrarapid metabolizers (UM, ultrarapid enzyme activity). It has been suggested that the genetically predicted metabolizer phenotype would be indicated with “g” (e.g., gPM) (Gaedigk et al. 2017), as also other factors, in addition to genetics, affect an individual’s metabolic capacity.

As stated previously, citalopram and escitalopram are metabolized mainly by *CYP2C19* but the *CYP2D6* (and to a lesser extent *CYP3A4*) enzyme also participates (Figure 4). Both *CYP2C19* and *CYP2D6* are highly polymorphic. Over 30 *CYP2C19* and over 100 *CYP2D6* allelic variants have been identified and inter-individual variation in coding genes leads to notable differences in drug metabolism capacity (Gaedigk et al. 2018).

Indeed, it has been shown that genetic variation in the *CYP2C19* gene has effects on citalopram and escitalopram concentration (Huezo-Diaz et al. 2012; Jukic et al. 2018; Mrazek et al. 2011; Rudberg et al. 2008b). It is possible that ultrarapid metabolism of these drugs would be one explanation behind insufficient treatment response and further therapeutic failure. Poor metabolism capacity, leading to accumulation of the drug, can also potentially predispose individuals to severe and even fatal adverse reactions, such as arrhythmias (U.S. Food and Drug Administration (FDA) 2012; Vincenzi and Lunetta 2015), in a dose-dependent manner.

In a recent publication by Jukic et al. (2018), it was revealed that escitalopram treatment failure measured by switch from escitalopram to venlafaxine or another SSRI was most common among escitalopram users defined either as UM or PM by *CYP2C19* genotype. Based on the results, it was concluded that genotype-guided dosing of escitalopram should be considered as routine clinical practice, as the fraction of patients who switch to another antidepressant was approximately 12% in reference group (with *CYP2C19* *1/*1 genotype) and approximately 30% in extreme phenotype groups (*CYP2C19* *17/*17 and *CYP2C19* loss-of-function/loss-of-function genotypes) (Jukic et al. 2018).

In *CYP2C19*, the gUM phenotype results from *CYP2C19**17, a regulatory polymorphism located in the 5’ flanking region of the *CYP2C19* gene (Sim et al. 2006). The loss-of-function gPM phenotype most commonly results from the *2-allele (de Morais et al. 1994b), although multiple other non-functional alleles (e.g., *3-allele (De Morais et al. 1994a) and *8-allele (Ibeanu et al. 1999)) have also been identified. Based on the measured drug plasma concentrations, the effect of the loss-of-function variant seems to be more notable compared to the effect of the increased-function variant (*CYP2C19**17) (Rudberg et al. 2008a). It is recommended that in the case of *CYP2C19* gPM, the citalopram/escitalopram dose should be decreased from the standard dose (Hicks et al. 2015).

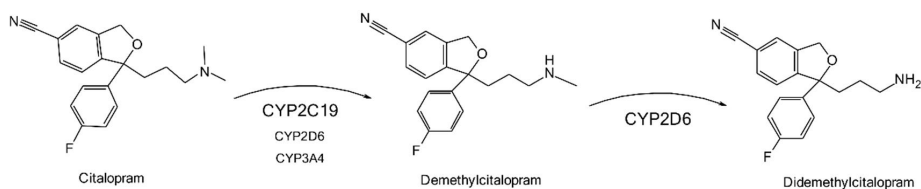


Figure 4 Metabolism of citalopram. The first metabolism phase from citalopram to demethylcitalopram is mainly catalyzed by the CYP2C19 enzyme. The CYP2D6 enzyme is responsible for the second demethylation step. In addition to demethylation, other modifications (N-oxidation, oxidative deamination, and glucuronide conjugation) are also possible (not shown) (Baselt 2000). Both S- and R-enantiomers of citalopram are metabolized by the same CYP enzymes. For simplicity, stereochemistry is not represented in this figure. The figure was produced with ChemDraw Professional 17.0 (PerkinElmer Informatics).

In addition to metabolic functions, the CYP2D6 and CYP2C19 enzymes have also been suggested to participate in endogenous functions as both are also expressed in brain tissue (Ahlner et al. 2010; Yu et al. 2003). Although some studies have found an association between brain expression and mental disorders, these associations are poorly understood (Jukic et al. 2017; Stingl et al. 2013).

P-glycoprotein

In addition to metabolic enzymes, transporter proteins also participate in pharmacokinetic processes (absorption, distribution, and excretion). P-glycoprotein (permeability glycoprotein, P-gp) functions as a gate-keeper transporter in multiple body barriers (Cordon-Cardo et al. 1989; Thiebaut et al. 1987). By pumping exogenous compounds out from inner compartments (e.g., from brain tissue to the blood circulation and from intestinal epithelial cell back to the gut lumen) it protects the body from harmful, exogenous compounds. P-gp has a large substrate spectrum that varies from endogenous steroids (Uhr et al. 2002) to exogenous substrates such as drugs (Uhr et al. 2003).

The pharmacological effect of a drug depends on its availability at the target site. Thus, when considering antidepressants, their ability to cross the blood-brain barrier (BBB) is one of the main considerations of drug design. Citalopram has been shown to be a substrate for P-gp (Uhr et al. 2008). Based on animal studies, the brain concentration of citalopram is affected by the *ABCB1* gene, which encodes P-gp. In *abcb1*⁻/*abcb1*⁻ knock-out mice, the citalopram concentration was higher compared to *abcb1*⁺/*abcb1*⁺ wild-type mice (Karlsson et al. 2013). In humans, the three most commonly investigated SNPs in the *ABCB1* gene are 1236C>T, 2677G>T/A, and 3435C>T. Of these, *ABCB1* 3435C>T was originally associated with decreased expression or function of P-gp, or both (Hoffmeyer et al. 2000). However, as this SNP does not cause an amino acid change, it was suggested that 3435C>T is in linkage disequilibrium with some other, functional SNP (Tanabe et al. 2001). Soon

thereafter, linkage disequilibrium between *ABCB1* 3435C>T, 2677G>T/A, and 1236C>T (Horinouchi et al. 2002; Kim et al. 2001) was confirmed. Despite the original findings, the effect of the 3435C>T, 2677G>T/A, and 1236C>T *ABCB1* polymorphism on P-gp expression remains controversial (Breitenstein et al. 2015; Owen et al. 2005).

As decreased function of the P-gp transporter protein may cause dose-dependent adverse effects, *ABCB1* gene polymorphisms have also received medico-legal attention (Neuvonen et al. 2011). Compared to CYP enzymes, altered P-gp function seems to have only a minor effect on plasma drug concentrations. However, it is possible that these minor differences become important in the brain.

3.5 ADVERSE DRUG INTERACTIONS IN CITALOPRAM-POSITIVE SUICIDES

In addition to genetic factors, drug interactions and adverse effects also explain poor treatment compliance. It was already suggested at the end of the 1990s by Druid et al. that forensic cases with suspicious toxicological findings should be investigated for genetic defects and drug interactions (Druid et al. 1999). According to the WHO, an adverse drug reaction is defined as “a response to a drug which is noxious and unintended, and which occurs at doses normally used in man” (World Health Organization 1972).

Drug interactions can be classified based on their mechanism, namely pharmacodynamic and pharmacokinetic interactions (Palleria et al. 2013). In pharmacokinetic interactions, the drug concentration is affected by some other drug. These interactions may occur in all ADME phases. In the metabolic phase, CYP-mediated interactions (induction or inhibition) are most common (e.g., inhibition of CYP2C19 by fluconazole). In the absorption and excretion phases, transporter-mediated interactions (e.g., inhibition of P-gp by verapamil) may occur. Compared to concentration-related pharmacokinetic interactions, pharmacodynamic interactions are usually additive (i.e., both drugs have similar effects). This means that interacting drugs do not affect the concentration of the other.

Citalopram is a very common finding in post-mortem toxicology (Launiainen and Ojanperä 2014) and has the potential for both arrhythmic and serotonergic adversities. When considering citalopram-positive suicide cases, these adversities are of great interest (Deshmukh et al. 2012; Musshoff et al. 1999) but are impossible to detect post-mortem (Vincenzi and Lunetta 2015). An example of a drug pair with a pharmacodynamic, arrhythmic interaction is citalopram and levomepromazine. Both drugs prolong the QT-interval of the heart (Ozeki et al. 2010; U.S. Food and Drug Administration (FDA) 2012). When these drugs are used simultaneously, the risk of serious arrhythmia leading to sudden cardiac death is increased. Serotonin syndrome (Sternbach 1991) may develop from simultaneous use of two serotonergic

antidepressants or concomitant use of e.g., citalopram and tramadol. Although serotonin syndrome has characteristic symptoms and is thus rather easy to recognize in medical care (Sternbach 1991), most of the medico-legally investigated deaths occur without eyewitnesses.

4 AIMS OF THE STUDY

The aim of this thesis was to investigate the role of pharmacogenetic factors in suicides of citalopram-treated persons. We hypothesized that genetic factors causing either insufficient response to treatment or adverse effects would be enriched in this extreme population of antidepressant users. In addition, the effect of a genetically predicted *CYP2C19* metabolizer phenotype on measured post-mortem citalopram concentrations and the role of adverse interactions in suicidal poisoning deaths was evaluated. Before genotyping the actual study population, the quality and quantity of FTA material used was characterized.

The detailed aims of the individual studies were:

- I. To investigate the time-dependent DNA degradation and its effect on the quality and quantity of DNA obtained from dried blood samples, preserved on FTA card matrix. Based on this study, the amount of the FTA sample used for the extractions and methods used for the pharmacogenetic analysis were determined.
- II. To investigate if *5HTTLPR*, rs25531, *MAOA-uVNTR*, and *HTR2B Q20** polymorphisms, previously associated with poor treatment response and aggressive behavior, are enriched among citalopram-positive completed suicides compared to citalopram-positive post-mortem controls. The effect of alcohol in suicide deaths was also evaluated.
- III. To assess if the *ABCB1* 1236T-2677T-3435T haplotype, associated with lower transport function of P-glycoprotein, is enriched among completed suicides of citalopram users.
- IV. To investigate the role of clinically relevant *CYP2C19* and *CYP2D6* polymorphisms and adverse drug interactions in citalopram-positive suicides.

5 MATERIALS AND METHODS

5.1 SAMPLES

Data mining and toxicological samples

The study population was searched from the post-mortem forensic toxicological database, which is maintained by the Laboratory of Forensic Toxicology, University of Helsinki (currently the Forensic Toxicology Unit, National Institute for Health and Welfare). The case inclusion was based on the positive citalopram finding measured from post-mortem blood (Rasanen et al. 2003). All toxicological analyses were performed from femoral blood samples containing 1% sodium fluoride. Samples were stored at +4°C after the autopsy.

Only cases investigated at the Department of Forensic Medicine, University of Helsinki were included in the study. Cases were further filtered by the defined MoD, suicide vs. other than suicide (excluding undetermined deaths). Control cases with MoD as accident were reviewed with caution. Controls were pairwise matched with cases by sex, age (± 10 years), and blood alcohol concentration (BAC) ($\pm 1\%$).

Dried post-mortem blood samples on FTA card

After study group formation, blood samples taken during autopsy and stored as dried blood spots on Whatman™ FTA Gene cards (GE Healthcare Bio-Sciences Corp., NJ, USA) were collected from the archive. Samples had been collected routinely during the autopsies in addition to all other forensic samples. After collection and initial drying, samples were stored at room temperature inside an envelope with desiccant pack and protected from light and humidity. As blood samples were missing for a few cases identified during the data mining (8/357), the study population eventually included 349 citalopram-positive suicide cases and 284 citalopram-positive control samples. The lower number of controls was due to the distortion of age distribution, suicides comprising younger individuals.

Prescription and purchase data

Individual toxicological findings cannot reveal whether the positive post-mortem finding is due to prescription-based usage or only random ingestion of drugs. We aimed to estimate prescription-based usage by purchase information. All medical purchases in individual pharmacies are entered into the register of national health insurance reimbursed prescription medicines. The register is maintained by Kela, the Social Insurance Institution of Finland. The registered variables include e.g., the unique personal identity number issued to all residents in Finland, which permits the linkage with autopsy data (data from death certificates and forensic toxicological database).

The prescription and purchase data were requested based on the findings from forensic toxicological analysis and the drug's reimbursability. The dates of prescription and purchase were requested for the following drug compounds specified with codes of the Anatomical Therapeutic Chemical (ATC) classification system (WHO Collaborating Centre for Drug Statistics Methodology 2018): No2 analgesic drugs (No2AA, No2AB, No2AC, No2AE, No2AJ, No2AX), No3 antiepileptic drugs (No3AE, No3AF, No3AG, No3AX), No5 psycholeptic drugs (No5AA, No5AB, No5AC, No5AD, No5AE, No5AF, No5AG, No5AH, No5AL, No5AN, No5AX, No5BA, No5BB, No5BE, No5CD, No5CF), No6 psychoanaleptic drugs (No6AA, No6AB, No6AG, No6AX, No6BA, No6CA), No7 other nervous system drugs (No7BC), Co7 beta-blocking agents (Co7AA, Co7AB), and M3 muscle relaxants (Mo3BC, Mo3BX). Requested prescription and purchase information covered 12 months preceding death.

5.2 LABORATORY METHODS

Before processing the actual samples (Studies II-IV), smaller set of samples (Study I) was extracted and quantified to characterize the DNA quantity and quality on the FTA cards. Samples for study I were selected among study population samples. We aimed to select four random samples from eight time points, covering the investigated time period of 1998 to 2013 ($n = 32$). This enabled an estimation of time-dependent degradation of the samples and more targeted selection of genotyping methods.

DNA extraction

Samples from study I were extracted identically to the actual samples. DNA extraction from FTA cards was performed with a semi-automated AutoMate Express™ Forensic DNA Extraction System using PrepFiler Express™ Forensic DNA Extraction Kit (Applied Biosystems™, ThermoFisher Scientific). The difference between actual and study I samples was the amount of input material used and the number of parallel samples.

In study I, FTA cards were pierced with a Harris Micro-Punch™-puncher (Ted Pella, Inc., Redding, CA, USA). Four 2.0 punches were used for each extraction. Each sample was extracted in triplicate. Based on the results, the amount of input material was increased for actual samples and approximately one fourth of the card's marked area was used for the DNA extractions (Studies II-IV). Actual samples were cut with scissors. In both DNA extraction sets, a 50- μ l final elution volume was used.

DNA quantification

In study I, DNA samples were quantified twice. Triplicates were first quantified to estimate internal sample variation and sample-to-sample variation. A second quantification was performed after pooling of triplicate samples. Actual samples were quantified only once. In all quantifications, a Quantifiler™ Human Plus (HP) DNA Quantification Kit (Applied Biosystems™) was used. This kit evaluates the total amount of human DNA and the quality, degradation, and inhibition level.

The DNA quality evaluation of Quantifiler HP is based on real-time polymerase chain reaction (RT-PCR). During the RT-PCR, two DNA fragments (a small [80 bp] and a large [214 bp] autosomal target) are amplified simultaneously. After amplification, the DNA degradation level is assessed by dividing the measured concentration of the small fragment with the concentration of the large fragment. This ratio is defined as the degradation index (Applied Biosystems (Thermo Fisher Scientific) 2017). A degradation index over one indicates poor amplification success of the large fragment compared to the small fragment, and hence degradation of the sample.

The kit also allows assessment of inhibition. This is based on amplification success of the internal positive control (IPC) included in the assay. Inhibiting agents present in a sample cause lower amplification success of the IPC. This is seen as a shift in the cycle threshold value (Applied Biosystems (Thermo Fisher Scientific) 2017).

All samples were analyzed with a 7500 Real-Time PCR system. Data analysis was performed with the Human identification (HID) Real-Time PCR Analysis Software v1.2 (Applied Biosystems™).

Genotyping

Study I

To elucidate the actual effect of time-dependent degradation on archived post-mortem DNA samples, samples were analyzed with a highly sensitive forensic assay and by amplifying the pharmacogenetically interesting *CYP2D6* gene.

Samples were first amplified using a highly sensitive short tandem repeat (STR) kit (GlobalFiler™ PCR amplification kit, Applied Biosystems™). This kit is routinely used for forensic case work samples. In this assay, the amplicon size varies between 75 to 444 bp. The samples were then amplified with a long-PCR reaction targeting the large (5.1 kb) genomic *CYP2D6* gene fragment (Sistonen et al. 2005). For these amplification reactions, DNA samples were normalized for 2 and 35 ng, respectively, based on the DNA concentration determined in a previous phase. The amplified DNA fragments were separated using a capillary electrophoresis (3500xL Genetic Analyzer, Applied Biosystems™) instrument (STRs) and the 2100 Bioanalyzer Instrument (Agilent Technologies) (*CYP2D6*). The results were analyzed with

GeneMapper® ID-X ver. 1.4 software and Bioanalyzer with 2100 Expert Software, respectively.

Studies II-IV

The genotyping methods used in studies II-IV were chosen based on the results from study I. In addition to study I, genotyping for studies II and III were performed in the Laboratory of Forensic Biology, Department of Forensic Medicine, University of Helsinki. Genotyping for study IV was performed in collaboration with Professor Mikko Niemi's research group, Department of Clinical Pharmacology, University of Helsinki.

5HTTLPR, MAOA-uVNTR, and rs25531 (Study II)

To analyze the length polymorphisms (5HTTLPR and MAOA-uVNTR), samples were normalized to 2 to 10 ng/μl DNA concentration based on the defined DNA quantity. Primer sequences used for the amplification were previously published by Nakamura et al. (Nakamura et al. 2000) and Sabol et al. (Sabol et al. 1998). For analysis purposes, the original primer sequences were modified to include fluorescent label. PCR-amplified fragments were separated with a 3500xL Genetic Analyzer with GeneScan™ 1200 LIZ® Size Standard for the fragment length determination. Interpretation of the results was performed with GeneMapper® ID-X ver. 1.4 software.

Correct allele calls for 5HTTLPR and MAOA-uVNTR were verified with Sanger sequencing of representative alleles. Sequencher v.4.10.1 software (Gene Codes) was used for data analysis.

After verification, the PCR products of the samples with a long l allele (homo- or heterozygote) of the 5HTTLPR were digested with the *MspI*-restriction enzyme to identify the rs25531 polymorphism. Digested samples were run along with a 500LIZ™ size standard with a capillary electrophoresis instrument and analyzed with GeneMapper® ID-X ver. 1.4. For details of 5HTTLPR, MAOA-uVNTR, and rs25531 genotyping, see study II.

HTR2B Q20* and ABCB1 polymorphisms (Studies II and III)

Genotyping of the *HTR2B* Q20* and *ABCB1* (1236C>T, 2677G>T/A, and 3435C>T) polymorphisms was performed with TaqMan Genotyping Assays (Applied Biosystems™). For genotyping, both custom TaqMan SNP Genotyping Assays (*HTR2B* Q20*, *ABCB1* 1236C>T, 2677G>T, and 3435C>T) and TaqMan Drug Metabolism Assay (*ABCB1* 2677G > A, assay ID C_11711720D_40) were used. Primer sequences for custom assays were obtained from the publications by Bevilacqua et al. (for *HTR2B* Q20*) (Bevilacqua et al. 2010) and Neuvonen et al. (for *ABCB1* 1236C>T, 2677G>T, and 3435C>T) (Neuvonen et al. 2011). Genotyping was performed according to the manufacturer's instructions and samples were analyzed using a 7500 Real-Time PCR System.

Genotyping of *CYP2C19* and *CYP2D6* polymorphisms (Study IV)

In study IV, samples were genotyped for the clinically relevant *CYP2C19* and *CYP2D6* polymorphisms and for *CYP2D6* copy number variation (CNV). Depending on the subsequent analysis, samples were normalized either to 5 ng/μl DNA concentration (TaqMan® Copy Number Assay and TaqMan® OpenArray™ with preamplification reaction, Applied Biosystems™) or to 30 to 50 ng/μl DNA concentration (TaqMan® OpenArray™ without preamplification reaction). Samples with DNA <30 ng/μl were preamplified prior to OpenArray reactions. Genotyping was performed according to the manufacturer's instructions with QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems™) and the obtained genotype data was interpreted using the following software: TaqMan® Genotyper Software ver. 1.3 (OpenArray results) and CopyCaller® Software ver. 2.1 (CNV results).

CYP2D6 copy number variation was primarily determined with the assay targeting exon 9 (Hs00010001_cn) of the *CYP2D6* gene. If the amplification success was poor, two additional CNV assays that targeted introns 2 (Hs04083572_cn) and 6 (Hs04502391_cn) of the *CYP2D6* gene were used. An assay targeting the human *RNase P H1* RNA gene was used as a reference assay.

In study IV, Finnish population samples were used as genotyping controls. The genotypes and demographic information for these samples was provided by Professor Mikko Niemi.

5.3 INTERACTION ANALYSIS

In study IV, all forensic toxicological findings detected among the suicide victims were searched for relevant adverse drug-drug interaction pairs or risk cumulations. Drugs used for resuscitation purposes (flecainide, lidocaine, and propofol) were excluded from the analysis, as use of these drugs is not intentional even though they appear in forensic toxicological analysis. The search was performed using the online database Riskbase/Inxbase v. Q4/2017-Q2/2018 (Medbase Ltd, Turku, Finland). The database grades the interaction pairs by clinical significance (A-D) and level of documentation (0-4). Only clinically significant interactions and risk cumulations with all levels of documentation (class Do-D4) were included in the study.

Identified interaction pairs were characterized as pharmacokinetic or pharmacodynamic and further divided into the following four groups: serotonin syndrome (SS), arrhythmias (A), sedation (SE), or other (O), including mixed cardiovascular and central nervous system adversities.

Cases were first analyzed as a whole and then at subgroup level by excluding cases with any single toxic drug concentration or blood/urine alcohol concentration $\geq 3\%$. Post-mortem femoral blood reference values were used to evaluate the toxic drug concentration level in post-mortem settings (drug concentration above post-mortem upper 95th percentile [mg/l])

(Launiainen and Ojanperä 2014). The suicidal intention of drug intake was evaluated from death certificates (circumstances of the death). The assessment of concurrent lifetime drug use vs. random ingestion of drugs was based on purchase information obtained from Kela (Figure 5).

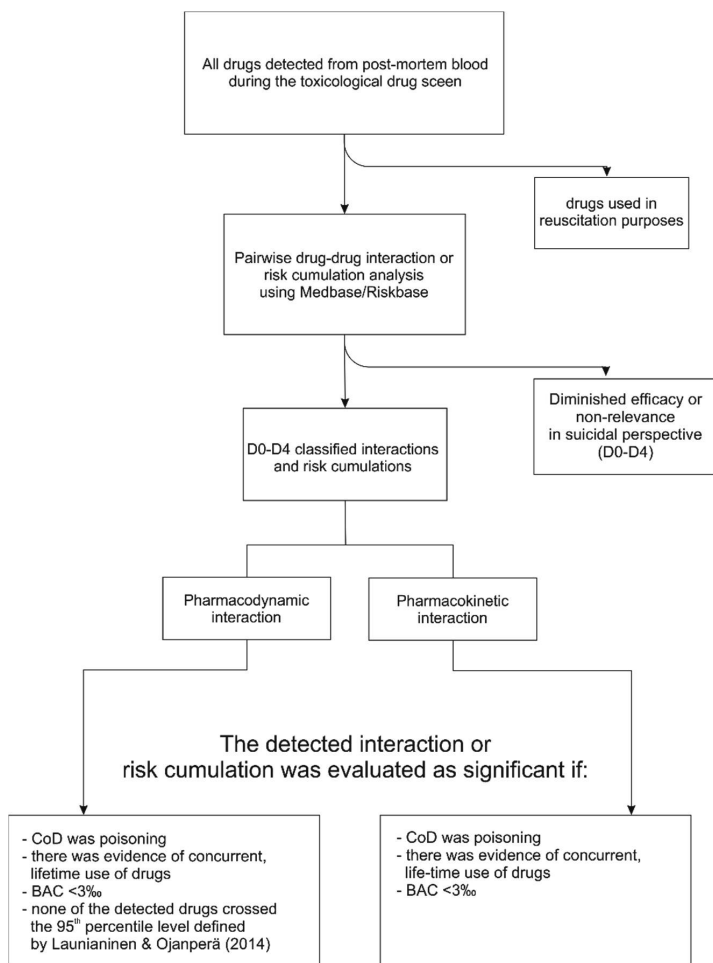


Figure 5 Drug interaction evaluation scheme.

5.4 STATISTICAL ANALYSIS

In study I, the effect of storage time on DNA yield and degradation was estimated by plotting the measured concentrations and DI values against the storage time. Statistical significance was evaluated with Pearson Correlation Coefficient using IBM SPSS Statistics software, version 22 (Armonk, NY).

For all genetic SNP and VNTR markers in studies II to IV, allele and genotype frequencies were estimated with Arlequin software version 3.5.1.2

(Excoffier and Lischer 2010). Departure from Hardy-Weinberg equilibrium (HWE) was tested using χ^2 -test. In the case of X-chromosomal *MAOA*, HWE was tested only among females. Cases and controls were tested separately with all markers. *ABCB1* haplotypes were inferred from genotype data using PHASE software version 2.1 (Stephens and Scheet 2005; Stephens et al. 2001). Summary statistics for age, sex, and measured post-mortem citalopram and alcohol concentrations were calculated with RStudio, versions 0.99.486, 1.0.153, and 1.1.447 (RStudio Team 2016 2016).

In studies II and III, binary logistic regression analysis implemented in Rstudio was used to estimate the association between genetic markers and MoD. Statistical significance was evaluated with 95% confidence intervals (CI). In study IV, Finnish population samples were used as controls, thus the differences between cases and controls were tested with χ^2 -test implemented in Rstudio. The statistical significance was evaluated by calculating binomial proportion 95% CIs for the frequency point estimates.

5.5 PERMITS AND ETHICAL STATEMENT

The study was approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District. The required permits for the scientific usage of autopsy material and records and prescription and purchase data were requested from the following responsible authorities: the National Institute for Health and Welfare, the Regional (Southern Finland) State Administrative Agency, and the Social Insurance Institution of Finland.

6 RESULTS AND DISCUSSION

6.1 POST-MORTEM MATERIAL IN PHARMACOGENETIC STUDIES (STUDY I)

It is known that post-mortem material may pose challenges for DNA analysis (Bar et al. 1988). The quality of DNA depends on post-mortem interval (Hansen et al. 2014), sampling site and tissue (Bar et al. 1988; Wheeler et al. 2017), and prevailing environmental conditions prior to sampling (Sorensen et al. 2016).

Since the 1990s chemically treated filter papers that protect attached DNA from nucleases and environmental factors have been available (GE Healthcare Life Sciences 2011). DNA technology used in forensic settings is optimized to overcome low amounts of DNA, the presence of inhibitors, and degradation (Calacal et al. 2015). However, it was not known if dried post-mortem blood samples preserved on FTA cards were suitable for other than highly sensitive forensic applications targeting small amplicons, and how long such samples can be stored for efficient DNA analysis in general.

In study I, we investigated how well DNA from post-mortem blood collected during autopsy and stored on FTA cards is preserved over a 16-year time period. This time period is equivalent to the preservation time of the post-mortem samples investigated in studies II to IV. The main result of the study was the following: DNA extracted from post-mortem blood dried on the FTA card matrix is rather well preserved over time. Although time-dependent degradation does exist, this mainly affects the methods that require large amounts of long, intact DNA fragments.

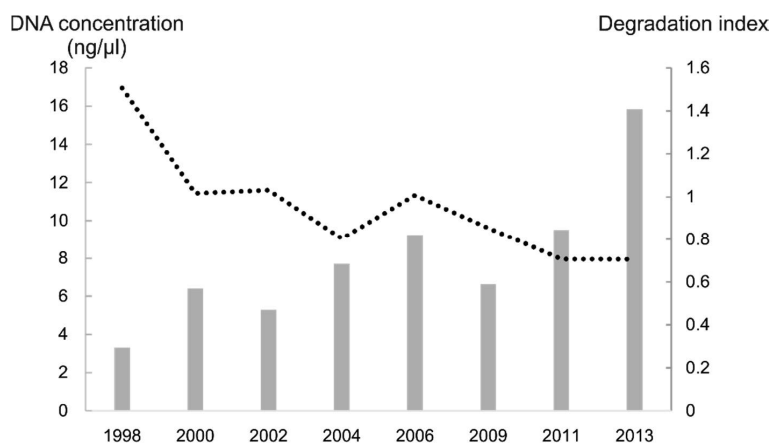


Figure 6 Quantification (primary axis) and qualification (secondary axis) results from pooled DNA samples. DNA concentration (grey bars) was shown to decrease and degradation increase (black dots) over the preservation time.

Extraction and quantification of sample triplicates revealed high variation both within and between samples. Internal variation (i.e., differences in DNA content between different spots on the same FTA sample) could be threefold (e.g., from 12 ng/ μ l to 44 ng/ μ l [SD 16.1]). The reason for this is unclear but may be due to post-mortem changes, such as formation of cell aggregates (Bar et al. 1988) occurring before the sample is applied to the card. It is possible that altered viscosity of the blood and cell aggregation leads to unequal distribution of the blood on the FTA card matrix. Across different samples, the mean DNA concentration varied from 2 ng/ μ l (as minimum) to 27 ng/ μ l (as maximum). None of the DNA samples showed traces of inhibition.

In pooled samples, despite few individual samples with high DNA concentrations, the overall trend showed that the older the sample was, the less (amplifiable) DNA was obtained. The same was true when samples were evaluated for degradation (Figure 6). In older samples, STR analysis showed a clear downward trend in peak heights of longer amplicons. This trend was not observed in more recent samples, where different-sized fragments amplified equally well (Figure 7). Analysis of *CYP2D6* amplification results confirmed the effect of degradation when analyzing longer DNA fragments.

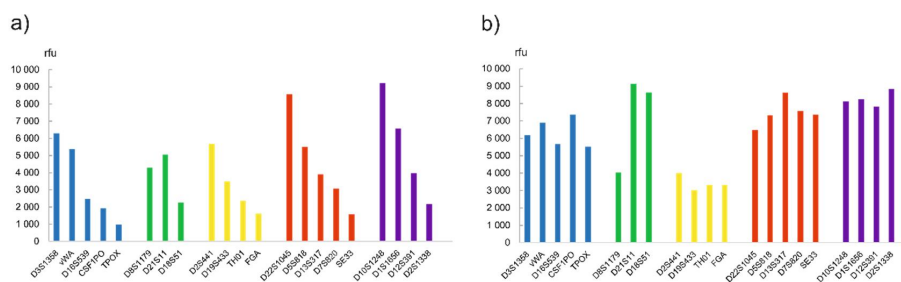


Figure 7 Comparison of STR profiles of two samples, one originating from 1998 (a) and the other from 2013 (b). In the GlobalFiler™ PCR amplification kit, 6-dye fluorescent chemistry is used to differentiate the same-sized DNA fragments from each other. Different colors used here correspond to the 6-dye system; blue (6-FAM™), green (VIC™), yellow (NED™), red (TAZ™), and purple (SID™). The 6th dye (LIZ™) is used for size standard (not shown here). Peak heights are presented in relative fluorescent units (rfu). The shortest locus included in each dye is shown on the left end and the longest on the right end. In the 1998 sample (a), the time-dependent degradation is evident. The longer the fragment is, the poorer is the amplification success (lower fluorescence intensity). In the 2013 sample (b), all fragment lengths seem to amplify equally well.

Based on the results, we concluded that proper quantification of post-mortem samples with RT-PCR based methods is essential, as the variation in DNA concentration and quality is high and the amount of sample is limited. Due to the internal variation, we also concluded that for research purposes it is more practical to cut a large piece from the FTA card with scissors rather than punch multiple discs with a puncher and lose the material between punched regions.

As both the STR and the *CYP2D6* analyses showed clear evidence of degradation, we decided to utilize TaqMan chemistry-based methods and highly sensitive capillary electrophoresis and RT-PCR instruments for consequent genotyping. To maximize amplification success, rather short (<400bp) amplicons were preferred in subsequent analyses if possible.

6.2 DEMOGRAPHICS OF CITALOPRAM-POSITIVE SUICIDE VICTIMS DIFFER FROM UNSELECTED SUICIDE POPULATION (STUDY II)

The number of suicide deaths in Finland have decreased significantly since 1990. The reasons for this are undoubtedly multifactorial. During the last three years the number has been rather constant at less than 800 cases per year (Official Statistics of Finland 2014; Official Statistics of Finland 2015; Official Statistics of Finland 2016b). Despite the overall decrease of medico-legal autopsies (Official Statistics of Finland 2016a), the Finnish legislation and the medico-legal CoD investigation practice assures that the decrease has had no effect on autopsy rates of suicide deaths. Thus, suicide statistics can be considered consistent and reliable. Compared to many other countries, the number of medico-legal autopsies in Finland is still rather high. In some countries, suicide statistics are skewed due to low medico-legal autopsy frequency (Ylijoki-Sorensen et al. 2014).

Among Finnish suicide victims, males are over-represented compared with females (3:1, respectively). Furthermore, males commit suicide more commonly using violent methods. However, these general observations made in previous studies (Hakko et al. 1998; Henriksson et al. 1993; Lahti et al. 2014) were not true among the citalopram-positive suicide cases studied here.

In citalopram-positive suicide victims, the observed male-female ratio was almost equal; 53% of suicides were committed by males and 47% by females (Figure 8). In addition, non-violent suicides were exceptionally common among males (52%). The reasons behind these observations are possibly due to the inclusion criteria used.

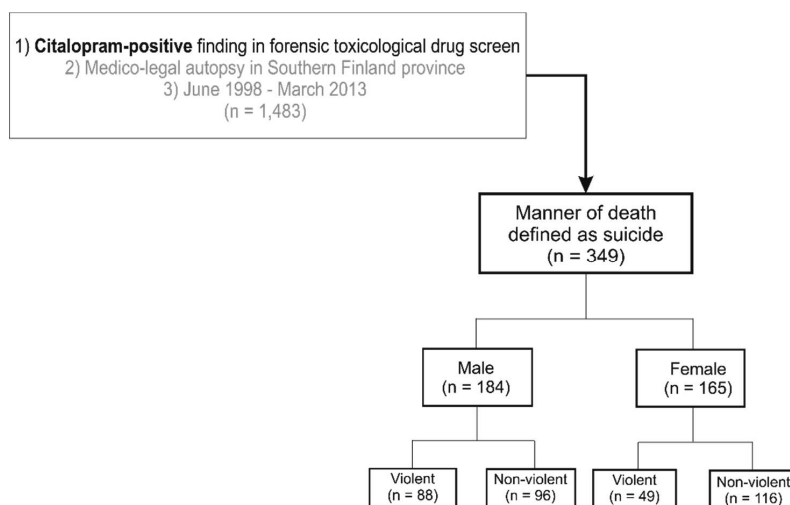


Figure 8 Inclusion criteria for the study group selection.

The cases were selected based on the positive post-mortem citalopram finding. Most of the cases (85%) had a valid citalopram/escitalopram prescription as well (Study III). Based on this, we deduced that citalopram-positive suicide victims are practically the same as citalopram-treated suicide victims.

The enrichment of females in our case population is probably because depressive disorders (often treated with citalopram) are more common among female suicide victims compared with males (Arsenault-Lapierre et al. 2004). It has also been questioned if the higher incidence of depressive disorders is biologically true or if females are only more willing to seek help (Kessler et al. 1981). Nevertheless, both explanations would lead to overrepresentation of females in the study material.

One factor affecting the suicide method used is the availability (Öhberg et al. 1995). Thus, as most of the individuals received medical treatment it is probable that they had easy access to drugs. This could explain the high number of drug poisonings (Öhberg et al. 1998).

6.3 ENRICHMENT OF THE EXTREME *CYP2C19* GENOTYPES – INDICATION OF A PHARMACOGENETIC EFFECT? (STUDY IV)

One of the main goals of the National Suicide Prevention Project in Finland was to investigate the role of mental health problems in suicide deaths with a special focus on depression recognition and adequate depression treatment. Suicides of young males also received special attention (Isometsä et al. 1994; Lönnqvist et al. 1993). There has been a notable decrease in the number of

suicides during and after the project. However, the rate is still rather high (Official Statistics of Finland 2016b). This raises the following question: were some risk factors unreachable with the approaches used during the project? Based on the general suicide statistics, one cannot conclude if the observed decrease has been due to decrease of violent, non-violent or both suicide types.

Pharmacogenetics entered the post-mortem field in the 1990s. It is expected that if pharmacogenetic factors affect antidepressant treatment success, this would be seen in our citalopram-treated suicide victims. Thus, by assuming that suicide completers, who despite antidepressant treatment commit suicide, are the same as those who will not benefit from antidepressant medication, it is possible that suicide mortality could be lowered further by taking account pharmacogenetic differences.

In study IV, by concentrating on citalopram pharmacokinetics we observed that the combined group of extreme gMPs (poor and ultrarapid metabolizers) of citalopram/escitalopram metabolizing *CYP2C19* were enriched among completed suicide cases compared to the Finnish general population. This observation is consistent with a recently published study (Jukic et al. 2018). In that study, escitalopram users with poor or ultrarapid metabolism capacity were observed to switch their antidepressant medication more often than users with extensive metabolism capacity. It was also observed that in gUMs the measured escitalopram concentration was under the therapeutic range. Ultrarapid metabolism capacity was concluded to be a probable reason for the switch. This gUM effect on citalopram concentrations could not be observed in our post-mortem study. However, it has been shown that the effect of the loss-of-function variant, causing poor metabolism capacity, is more notable compared to the effect of the increased-function variant (Rudberg et al. 2008a). Also, the dosing recommendations of citalopram/escitalopram consider only the *CYP2C19* gPM phenotype (Hicks et al. 2015). Thus, it is possible that even though the concentration of citalopram would be slightly lower in suicide cases determined as gUM, small genetic effects in drug concentration are covered by post-mortem redistribution after the death. In the case of citalopram, the ratio between post-mortem blood and ante-mortem plasma concentration is 3.6 (Launiainen and Ojanperä 2014), meaning that citalopram has rather high redistribution characteristics after death.

Instead, we made an interesting medico-legal observation. The effect of the genetically predicted *CYP2C19* phenotype on measured post-mortem citalopram concentration was evaluated among violent suicide victims grouped by *CYP2C19* phenotype (gPM, gIM, gEM or gUM). It was observed that in the gPM group, the measured citalopram concentration was equal to the 90th percentile citalopram concentration level defined by Launiainen and Ojanperä (Launiainen and Ojanperä 2014) (Figure 9). The purpose of the compilation by Launiainen and Ojanperä was to provide guidelines for the interpretation of forensic toxicological results, as interpretation of post-mortem drug concentrations often poses a challenge for forensic pathologist. Upper percentile concentrations were defined as a possible indication of drug

overdose. Based on the results shown here, it seems that CYP metabolizer status may affect the interpretation of forensic toxicological results. However, the observed trend needs to be investigated further in a larger sample set and with other drug compounds.

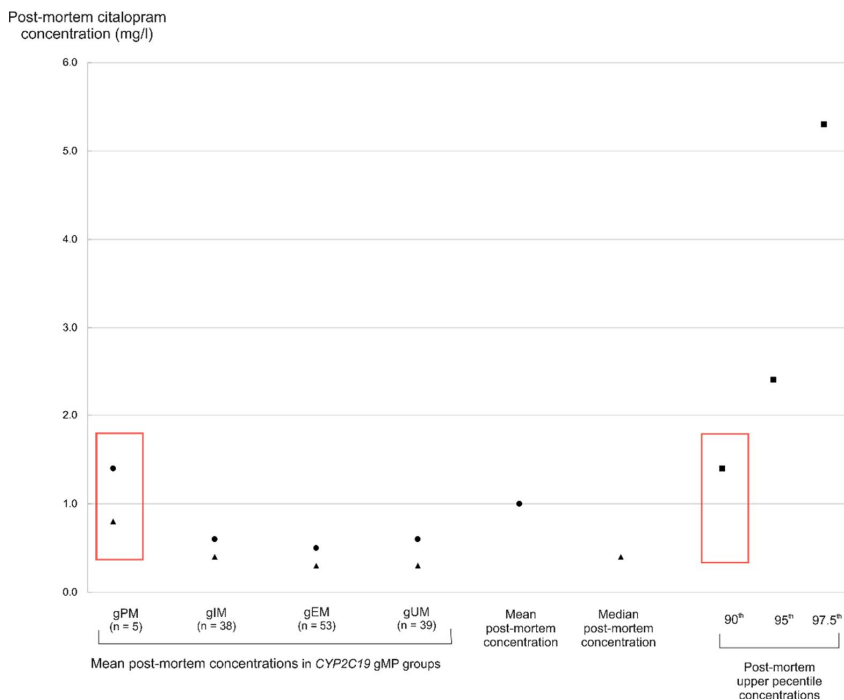


Figure 9 Post-mortem mean (●) and median (▲) citalopram concentrations grouped by *CYP2C19* phenotype (genotype-predicted) and post-mortem mean, median and upper percentile (■) citalopram concentrations defined by Launiainen and Ojanperä (2014). As highlighted with red boxes, the citalopram concentration in the gPM group is equal to the 90th percentile citalopram concentration level (Launiainen and Ojanperä 2014). The figure has been modified from (Rahikainen et al. 2018).

Based on these results, it seems that the citalopram/escitalopram treatment response is affected by the *CYP2C19* genotype. As similar result was already obtained from another study (Jukic et al. 2018) as well, it appears that pharmacogenetic analysis of depressed patients, especially those with suicidal thoughts, could be recommendable prior to initiation of a drug treatment. However, it seems that citalopram treatment response is only affected by pharmacokinetic factors, as no association with the *5HTTLPR* genotype (pharmacodynamics) was found in study II. However, the expression level of this transporter may have an effect on the stress tolerance of a depressed individual (see below 6.4.2).

6.4 DIFFERENCE IN SUICIDE RATIO BETWEEN MALES AND FEMALES (STUDIES II-IV)

Sex differences in suicide statistics are an interesting phenomenon (Freeman et al. 2017). Based on the national suicide statistics (Figure 1), it seems that although the number of female suicide victims has consistently been lower compared to males, females have also been less affected for the preventive acts established. In all studies (II-IV), suicide cases were first analyzed as a whole and after stratified by sex and suicide method used.

6.4.1 NON-VIOLENT SUICIDE; INTENTIONAL OR ACCIDENTAL DRUG POISONING? ARE FEMALES WITH CERTAIN *ABCB1* AND *CYP2D6* GENOTYPES MORE VULNERABLE TO ADVERSE DRUG EFFECTS? (STUDIES III-IV)

In studies III and IV, enrichment of the *ABCB1* 1236T-2677T-3435T haplotype and *CYP2D6* gPM phenotype was observed among female suicide victims and further among the non-violent subgroup of females. Both of these defective genotypes cause accumulation of substrate drugs (Johns et al. 2002; Koski et al. 2007). Enrichment of poor functioning genotypes in intoxication deaths could suggest that females carrying these haplotypes or phenotypes are more vulnerable to concentration-related drug adversities. It is possible that female suicide attempters with a susceptible genetic background are in danger of suicide death that is actually unintended. Indeed, attempted suicides seem to be more common among females (Freeman et al. 2017; O'Loughlin and Sherwood 2005). This could explain the lower decrease in suicide rates compared to males and “resistance” for the preventive acts.

However, our case-by-case evaluation revealed only one *CYP2D6* gPM case with elevated concentration of a *CYP2D6*-dependent drug (amitriptyline) and unclear suicide intention. Thus, no clear explanation for the observed enrichment was attained. It also remains unclear why the same genotypic effect was not observed among males who committed suicide by drug poisoning.

Both *ABCB1* and *CYP2D6* have also been associated with suicidal deaths in other studies (Boiso Moreno et al. 2013; Zackrisson et al. 2010). However, the biological mechanism behind the observed associations remains unclear. Zackrisson et al. (2010) found enrichment of *CYP2D6* gUMs among suicide victims (intoxications excluded) compared with those whose MoD was defined as natural. It was concluded that this enrichment might be linked with insufficient response to antidepressant treatment. However, no information on the medical histories of these individuals was available (Zackrisson et al. 2010). In the Finnish population, the frequency of *CYP2D6* gUM is rather high (7.2%) (Pietarinen et al. 2016). In study IV, the frequency in citalopram-treated suicide victims (7.7%) was observed to be close to the general

population frequency. Thus, based on our results it seems that *CYP2D6* is not related to suicidality in general or personality traits at least in citalopram-related suicides (Ahlner et al. 2010; Yu et al. 2003). However, it is possible that the ultrarapid phenotype of *CYP2D6* could explain the insufficient response to treatment. For this, suicide victims treated with primarily *CYP2D6*-dependent antidepressant should be investigated.

6.4.2 ASSOCIATION BETWEEN *5HTTLPR* GENOTYPE AND SUICIDE INTENTION, OBSERVATION FROM SUICIDE METHOD USED (STUDY II)

The results from study II highlighted that the citalopram-positive suicide population is different than the unselected suicide population. However, it also seems that the population investigated is internally heterogeneous. This may be explained by the fact that although citalopram is a first-line antidepressant to treat depression, it is also used for other purposes such as treatment of anxiety. As briefly mentioned in section 6.3, no association between serotonin transporter variation and risk of suicide was detected when the suicide population was analyzed as a whole. Thus, it can be concluded that citalopram treatment success does not depend on the *5HTTLPR* genotype. However, stratification by sex and suicide method used revealed that the low-functioning *5HTTLPR* ss genotype possess a risk for violent suicide among males. This was an interesting finding as it is possible that the *5HTTLPR* genotype modifies the response to stress, which in turn works as a trigger for suicidal act (Caspi et al. 2003), or relates to the depth of depression and high suicidal intent, which is indirectly seen as a violent suicidal act.

No association between the risk of suicide and two other polymorphisms investigated in study II (*MAOA-uVNTR* and *HTR2B Q20**) was detected. However, the BAC was only found to be a risk factor for non-violent but not violent suicide. This is quite surprising. The association between non-violent suicides and alcohol is clear as alcohol potentiates the sedative effects of multiple drugs (e.g., opioids and benzodiazepines). However, the missing association between alcohol and violent deaths is more difficult to explain. Alcohol is frequently associated with violent suicides (Lunetta et al. 2001; Ohberg et al. 1996) as it increases self-confidence and may lower the threshold of suicide. Furthermore, suicide cases with underlying alcohol use disorder (AUD) are more impulsive and aggressive compared to suicides without AUD background (Chachamovich et al. 2012). But this was apparently not the case in our material. It is possible that genetic factors associated with aggressive and impulsive behavior when under the influence of alcohol (Tiihonen et al. 2015; Tikkanen et al. 2015) do not have a role in violent acts committed without alcohol. Impulsive violence against others might also be a different phenomenon than violence against oneself (Jokinen et al. 2017). However, this is only speculation, as we did not have ante-mortem patient records and

clinical diagnoses of suicide victims and thus knowledge of underlying AUD. Overall, the missing background information is a clear limitation of all studies included in this thesis.

6.5 ADVERSE DRUG INTERACTIONS IN FATAL DRUG POISONINGS (STUDY IV)

Polypharmacy is common phenomenon in forensic toxicology, especially in suicidal intoxications (Ohberg et al. 1996). Thus, uncovering adverse drug interactions and ADRs is not surprising as such.

In study IV, six pharmacokinetic and 208 pharmacodynamic interactions or risk cumulations were detected when the suicide population was analyzed as a whole. The low number of pharmacokinetic interactions was consistent with a previously published study (Launiainen et al. 2009). The number of pharmacodynamic interactions seems at first very high. Considering that pharmacodynamic interactions/risk cumulations are additive and a single, high (toxic) drug concentration can overrun the effect of an interaction, the relevance of an interaction as a contributory factor to poisoning death must be interpreted cautiously. However, the role of pharmacodynamic interactions should be kept in mind as in 8% of all drug poisoning deaths, a drug-drug interaction most probably contributed to the fatal outcome.

Drug adversities may lead to fatal outcome if a person is susceptible for certain type of adversity due to genetic background (e.g., congenital long QT syndrome (Lunetta et al. 2002)) or liver impairment (Vincenzi and Lunetta 2015). Pharmacodynamic interactions can be especially difficult to recognize as drug concentrations are not usually particularly high. As discussed in a previous publication (Launiainen et al. 2009), the role of interaction is rarely recognized by a forensic pathologist, even though the drugs involved were recognized to have contributed to death. This observation was also confirmed in study IV.

It is important to develop tools for better identification of potentially fatal drug combinations. With database-aided, semi-automated identification, a forensic pathologist could more readily interpret potential interactions in a particular case. In addition to interactions, toxicological drug findings in which a drug's metabolism is also affected by pharmacogenetic variation could be automatically highlighted in the future to alert the forensic pathologist of a potential need for ancillary (genetic) testing.

7 CONCLUSIONS

In Finland, forensic toxicological analysis is performed in most (>70%) medico-legal autopsies. Traditional forensic genetic analysis concentrates on crime scene samples, identification of human remains, kinship analysis, and paternity testing. Despite promising findings, post-mortem pharmacogenetics — an integrative discipline between forensic genetics and toxicology — is not routine practice among medico-legal CoD investigations.

Although most medico-legal cases are solved without pharmacogenetic testing, it is important to recognize in which types of cases genotype analysis would likely give relevant information for the CoD investigation. Increased drug concentration may be due to multiple causes, such as intentional intake, tolerance, pharmacokinetic interaction, or pharmacogenetic defect. Although pharmacogenetic variation explains a rather small part of increased drug concentrations, as shown in this thesis, it can be an important factor in an individual case. As it is not cost-effective to genotype all cases, comprehensive research is needed to be able to trace potential cases, in which pharmacogenetics testing may open a new interpretation of the CoD or the MoD.

In this study, a combined group of extreme *CYP2C19* phenotypes was observed more frequently from citalopram-positive suicides compared with population controls. It is thus possible that altered citalopram metabolism due to pharmacogenetic factors has an adverse effect on treatment outcome. In addition, a trend between the genetically predicted *CYP2C19* phenotype and post-mortem citalopram concentration was discovered, although no definite conclusion could be drawn due to the rarity of the *CYP2C19* poor metabolizer phenotype. This finding needs to be investigated further, as without knowledge of the underlying genotype an increased drug concentration can be falsely interpreted as intentional drug intake. This study also showed that in addition to post-mortem pharmacogenetics, studies on drug interactions deserve more attention in the realm of medico-legal autopsies. Lastly, we have shown that post-mortem genetic studies are achievable from samples stored for more than a decade. In conclusion, post-mortem pharmacogenetics has shown potential both in clinical practice and forensic medicine, and more effort and resources should be invested in this field in the future.

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Anna-Liina Rahikainen

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